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# Macronutrient deprivation modulates antigen trafficking and immune recognition through HSC70 accessibility

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## Abstract

B lymphocytes exploit macroautophagy to capture cytoplasmic and nuclear proteins within autophagosomes. Fusion of autophagosomes with lysosomes and endosomes facilitates content proteolysis, with the resulting peptides selectively binding MHC class II molecules (MHCII) which are displayed for recognition by T lymphocytes. Nutrient deprivation or stress amplified this pathway, favoring increased MHCII presentation of cytoplasmic antigens targeted to autophagosomes. By contrast, this stress diminished MHCII presentation of membrane antigens including the B cell receptor (BCR) and cytoplasmic proteins that utilize the chaperone-mediated autophagy pathway. While intracellular protease activity increased with nutrient stress, endocytic trafficking and proteolytic turnover of the BCR was impaired. Addition of macronutrients such as high-molecular mass proteins restored endocytosis and antigen presentation, evidence of tightly regulated membrane trafficking dependent on macronutrient status. Altering cellular levels of the cytosolic chaperone HSC70 was sufficient to overcome the inhibitory effects of nutritional stress on BCR trafficking and antigen presentation. Together, these results reveal a key role for macronutrient sensing in regulating immune recognition and the importance of HSC70 in modulating membrane trafficking pathways during cellular stress.

## Introduction

Nutrition has a profound influence on host immunity as seen by connections between immune deficiency and malnutrition (*1*). Protein malnutrition and serum protein levels are linked to increased susceptibility to infection (*2, 3*). In children, protein deprivation results in reduced Ab responses and B cell expansion with bacterial infection (*4, 5*). These studies indicate protein malnutrition impairs B and T cell mediated immunity, although the molecular mechanisms remain poorly defined.

Induction of high-affinity, long-lasting humoral immunity depends on B and T cell interactions. Intracellular trafficking pathways play a key role in promoting B and T cell contact necessary for adaptive immune responses. Ags are delivered into the endosomal network by various transport routes including receptor-mediated uptake, macroautophagy (MA), and chaperone-mediated autophagy (CMA) (*6, 7*). While endocytosis of exogenous

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Ags promotes the classical MHCII Ag presentation pathway, MA, bulk autophagy, and CMA, a selective form of autophagy dependent on HSC70, play critical roles in MHCII presentation of intracellular Ags (*7*).

To enhance Ag uptake B cells express membrane-bound Ig, which associates with adaptor proteins to form the BCR (*8*). Surface BCR, alone or complexed with Ag is constitutively internalized into endosomes. Here, cathepsins digest the BCR and Ags yielding peptides for presentation by MHC class II molecules (MHCII) (*9, 10*). MHCII are guided to endosomes by invariant chain, which is proteolytically released by cathepsins (*11*). A molecular editor, HLA-DM, then removes the invariant chain fragment CLIP from MHCII, facilitating antigenic peptide binding (*12*). The resulting MHCII:peptide complexes as well as some co-stimulatory molecules recycle from endosomes to the cell surface to engage T cells. Thus, trafficking of BCR:Ag complexes to endosomes promotes MHCII Ag presentation and enhances B and T cell interactions needed for Ab class switching and affinity maturation (*13, 14*).

In response to some stresses, cells upregulate or downregulate transport pathways such as MA, CMA, and endocytosis to maintain homeostasis. During nutrient stress, cells initially upregulate MA coupled with later changes in CMA to promote survival and to salvage critical building blocks (*15, 16*). Whether alterations in the activity of these pathways during nutritional stress influence immune recognition has not been well explored.

In B cells, macronutrient stress induced by protein deprivation impaired BCR and Ag trafficking altering MHCII Ag presentation. A shared requirement for the conserved heat shock protein, HSC70 was demonstrated to maintain these B cell functions during nutrient stress. These studies reveal macronutrient sensing in B cells modulates Ag trafficking and presentation, further connecting host nutrition with adaptive immunity.

## Materials and Methods

### Cells and Ag Presentation Assay

Human B-lymphoblastoid cell lines referred to as B cells or B lymphoblasts, including PriessGAD and FrevSMA were maintained in IMDM with 10% FCS (*17*). Human peripheral blood B cells were isolated using Ficoll gradients and CD19 magnetic beads (Milltinyi Biotech) followed by culture in RPMI 1640 media with 10 % FBS. Institutional approval for human blood collection was obtained for this study. For treatment without serum, cells were washed in HBSS and incubated in IMDM or RPMI 1640. PriessGAD cells were transduced to express influenza A matrix protein 1 (MP1) fused to the autophagy protein LC3 (plasmid a gift from C. Münz, University of Zurich) (*18*). Ectopic expression of human HSC70 in PriessGAD was previously described (*19*). T cells recognizing a BCR light chain ($Ig_{\kappa\ 88\text{-}203}$) epitope, an epitope from MP1 (provided by David Canaday, Case Western Reserve University), an epitope from MHC class I heavy chain or the $GAD_{273\text{-}285}$ epitope were maintained as reported (*14*). For analysis of Ag presentation, B cells were cultured in media +/− serum or in serum-free media with BSA. B cells were then fixed and co-cultured with epitope specific T cells or pulsed with 10 uM peptide for 6 h prior to incubation with epitope specific T cells. T cell activation was detected using an Il-2 bioassay

and HT-2 cells (*20*). To facilitate the comparison of results using human B lymphoblasts, the average value of T cell activation detected using control serum-cultured cells from three independent experiments was calculated and set to 1 in each study. For each experimental condition, the average T cell response was calculated from individual replicate experiments and plotted as a relative value compared with the average T cell responses observed using control cells, the latter referenced above as equal to 1.

### Immunoblots

Cell lysates from B cells cultured +/− serum were resolved by SDS-PAGE for immunoblot analysis of LC3II (Cell Signaling), BCR (Biosource), DR (DA6), invariant chain (PIN1.1), GAD (Sigma, GAD65/67), MP1 (AbD Serotech) or GAPDH (Millipore). Densitometry using ImageJ (NIH) was used to quantitate protein levels. Protein densitometry was normalized to the GAPDH or MHCII loading controls and the fold change determined relative to the control cells cultured with serum. For protein turnover, B cells were cultured in 10 ug/ml cycloheximide +/− serum prior to immunoblot analysis.

### MA Flux

Upon maturation and acidification of autophagosomes, LC3II protein within these vesicles is degraded. B cells were cultured +/− serum, +/− 50 ug/ml choloroquine (CQ), harvested and lysed for immunoblot and densitometry analysis. CQ is added to block autophagosomal degradation of LC3II. The relative increase in endogenous cellular LC3II levels detected with addition of CQ is a measure of MA flux. Basal cellular levels of LC3II/GAPDH were subtracted from LC3II/GAPDH levels accumulating with CQ treatment and normalized to serum treated cells (*21*).

### Flow Cytometry

B cells were harvested after an incubation +/− serum, fixed and stained for surface markers: DR (BD Pharmingen), CLIP (BD Pharmingen), CD45R (eBioscience), transferrin receptor (TfR) (B3/25) or BCR (Jackson Immunoresearch). To monitor surface levels of GM1 ganglioside, cells were incubated with 10 ug/$10^6$ cells biotin-conjugated cholera toxin (Life Technologies) prior to staining with an anti-biotin-PE secondary Ab. For intracellular staining, B cells were permeabilized in 0.1% saponin prior to DM (BD Pharmingen) or BCR (Jackson Immunoresearch) staining. Isotype matched Abs were used as controls. The average mean fluorescent index (MFI) of three independent experiments was determined for control cells cultured with serum. The fold change relative to this average was graphed.

### Protease Assays

Here, two separate assays were used to detect cellular cathepsin activity. In a static assay with cell lysates, cathepsin activity was monitored using plate-based fluorometry to detect cleavage of specific substrates: ZArgArgNMec for cathepsin B, ZPheArgNMec for cathepsin L, and ArgNMecHCl for cathepsin H (Sigma) (*22*). For real time analysis of cathepsin activity within the endosomal network of live cells a flow cytometric assay was used. Here, Magic Red™ Cathepsin B and L Kits (Immunochemistry Technologies) were used (*23*). After 11 h of treatment +/− serum, B cells were incubated one hour with 6 uM of

membrane permeable Magic Red protease substrates followed by flow cytometric analysis to detect substrate cleavage.

The Proteasome-Glo$^{TM}$ Chymotrypsin-like Cell-Based Assay (Promega) was used according to the manufacturer's instruction to assess proteasome activity in control cells cultured with serum and after 6 or 12 h serum deprivation (*24*). The average protease activity from three independent experiments for control cells feed with serum was determined and the fold change relative to this average was graphed.

### Endocytosis Assay

Specific endocytosis of the BCR, MHCII and the TrF was monitored by biotinylating B cell surface proteins using EZ-link Sulfo-NHS-SS-Biotin (Thermo) (*25*). Biotin-labeled cells were incubated 30 min at 37°C in media +/− serum, or in serum-free media with BSA. After the incubation, a glutathione solution was used to remove residual biotin-label from surface proteins. Cells were lysed and cell surface protein endocytosis determined using ELISA plates coated with anti-human IgG Ab (Jackson Immunoresearch, used at 2 ug/mL) for the BCR, anti-MHC class II 37.1 Ab (L. Wicker, used at 1ug/mL) or anti-TrF B3/25 Ab. Endocytosis was graphed as a percent of the total surface expression for each protein.

### Subcellular Fractionation

To determine if serum starvation altered GAD intracellular localization, membrane and cytoplasmic fractions were separated using a method previously published (*26*). PG cells were cultured with or without serum for 12 h, harvested, and lysed using the Balch homogenizer. Differential ultracentrifugation was then used to isolate membrane and cytosolic fractions for immunoblot analysis.

### Macronutrient Supplementation of Media

Serum was fractionated using Centricon concentrators with cut-offs of 30kDa and 10kDa according to manufactures instructions (Amicon). Macromolecules retained by the 30 kDa filter (>30 kDa fraction), and molecules flowing through filters were used as <30 kDa and <10 kDa fractions, respectively. Cells were cultured in IMDM media supplemented at 10% with the various serum fractions for 12 h before harvest.

Alternatively, cells were cultured in serum-free media supplemented with defined macromolecules including 1% (wt/vol) BSA, ovalbumin (OVA), hen egg lysozyme (HEL), or dextrans (70 kDa, 40 kDa, or 10 kDa). After 12 h, these cells were analyzed by flow cytometry.

### Osmolarity and Viscosity

Analysis of the osmolarity and viscosity of media samples with and without serum, BSA or dextran addition was conducted by a commercial laboratory ACTA using using USP 37/NF 32 methodology (Burbank, CA).

### Co-Immunoprecipitation

B cells were cultured in media +/− serum or in serum-free media supplemented with 1% (wt/vol) BSA for 12 h. Cells were lysed in 10 mM Tris pH 7.4, 150 mM NaCl, 1% *N*-octyl-β-glucopyranoside with protease inhibitor (Sigma-Aldrich) for 20 min on ice. Lysates were centrifuged at 14,000 rpm for 10 min. Normal rabbit serum (1:1000) and Protein G-Sepharose were added to lysate at 4°C for 30 min as a preclear step followed by removal of the Sepharose by centrifugation. GAD Ab (GAD6, Sigma) or an isotype matched control Ab were added to co-precipiate associated proteins. These Ag-Ab complexes were resolved on 10% SDS-PAGE and analyzed by immunoblotting. The ratio of HSC70/GAD was set to 1 in control serum-treated cells and the fold change relative to this ratio was graphed for each experimental condition.

### Statistics

Statistics were determined using GraphPad Prism 6.0 (GraphPad Software, Inc.). Data depict mean from 3 or more independent experiments, +/− SEM. P values less than 0.05 were considered statistically significant with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

## Results

### Nutrient Deprivation Alters MHCII Antigen Presentation

To elucidate the effects of macronutrient deficiency on MHCII presentation, B cells were cultured in serum-free media rich in amino acids, simple carbon sources and vitamins but lacking complex macromolecules. In vivo and in vitro, cellular deprivation of serum proteins is known to induce MA, a process by which LC3II decorated autophagosomes engulf cytoplasmic molecules for degradation and in some cases immune recognition. MHCII presentation of epitopes derived from a cytoplasmic Ag, influenza matrix protein 1 (MP1) that is targeted to autophagosomes by linkage to LC3, was increased in cells grown in serum-free media compared to serum treated cells (Fig. 1A). Cellular levels of MA or the MA flux was monitored by tracking the expression of the endogenous LC3II protein in response to nutrient stress with and without chloroquine. As expected, MA flux was amplified in cells grown without serum (Fig. 1B). Although MHCII (HLA-DR) surface levels and MHCII presentation of a synthetic peptide derived from the BCR were unchanged by serum deprivation, MHCII presentation of endogenous BCR epitopes was significantly inhibited (Fig. 1C-E). HLA-DM levels and editing of MHCII:CLIP complexes were unchanged by serum deprivation (Fig. 1E). Furthermore, cellular levels of MHCII and invariant chain were overall unchanged (Fig. 1F and Supplemental Fig. 1A). Thus, serum deprivation in B cells stimulated Ag presentation by MA while reducing MHCII display of self-epitopes derived from a membrane Ag the BCR.

### Nutrient Deprivation Alters Antigen Degradation

To elucidate the mechanism by which serum deprivation altered epitope selection, MP1 and BCR protein expression was assessed. While cellular MP1 protein levels dropped with serum deprivation (Fig. 2A), BCR protein levels dramatically increased (Fig. 2B). To

determine whether these alterations were due to changes in Ag synthesis or degradation, B cells were treated with a protein synthesis inhibitor, cycloheximide. MP1 protein levels decreased quickly with serum deprivation regardless of cycloheximide treatment, consistent with MA-induced degradation (Fig. 2A). Conversely, BCR protein levels rose irrespective of cycloheximide, consistent with impaired BCR degradation (Fig. 2B). Transcripts for BCR and MHCII components were unchanged by serum deprivation, further pointing to perturbations in cellular protein degradation pathways (Supplemental Fig. 1B and C).

To determine if disruption of BCR degradation was due to altered endosomal and lysosomal protease activity, cathepsin B, L and H activity were determined in lysates from B cells cultured with or without serum. Serum deprivation led to a significant increase in total cellular cathepsin B and L activity, but cathepsin H remained unchanged (Supplemental Fig. 1D). Furthermore, a real-time flow analysis of cathepsin proteolysis using membrane soluble-substrates in living B cells, revealed increased endo/lysosomal cathepsin B and L activity in response to serum deprivation (Fig. 2C and D). Furthermore, serum deprivation increased cathepsin B and L activity in freshly isolated human peripheral blood B cells from multiple donors (Fig. 2E). The reduced BCR turnover in the presence of increased cathepsin activation in this analysis pointed to possible alterations in BCR trafficking during serum macronutrient depletion.

## Nutrient Deprivation Impairs Endocytosis and Trafficking of the BCR

Analysis of BCR trafficking revealed a 2-fold increase in BCR surface levels in B cells cultured without serum (Fig. 3A). Total BCR expression increased with serum deprivation, along with the ratio of surface:total BCR expression (Fig. 3B), consistent with an accumulation of cell surface BCR. Surface BCR distribution increased more dramatically in peripheral blood B cells incubated without serum (Fig. 3C and D). Similarly, serum deprivation significantly enhanced surface levels of the transferrin receptor (TfR), which also utilizes the clathrin-mediated endocytosis pathway (Supplemental Fig. 1E). Interestingly, serum deprivation did not alter surface expression of CD45R nor the ganglioside GM1, neither of which use clathrin-mediated endocytosis for internalization (Supplemental Fig. 1F and G). Furthermore, specific internalization or endocytosis of the BCR was significantly impaired in response to serum deprivation of B lymphoblasts (Fig. 3E) as well as in peripheral blood B cells (Fig. 3F). To further assess the effect of serum deprivation on clathrin-mediated endocytosis, internalization of surface MHCII and TfR were monitored in peripheral blood B cells. Endocytosis of these molecules was slightly impaired with cellular serum deprivation (Fig. 3F). These studies pointed to a role for serum nutrients in regulating clathrin-mediated endocytosis.

## Macronutrients Restore Trafficking and Antigen Presentation

Serum is macronutrient rich containing proteins, lipids and complex carbohydrates. To determine if macronutrients played a role in alterations in BCR trafficking, serum components were fractioned based on molecular mass. Internalization of BCR was reduced with serum deprivation or upon cultivation of B cells with serum components smaller than 10 kDa. Serum fractions with higher molecular mass components (>10kDa) were more effective at restoring surface BCR levels (Fig 4A).

To determine if specific macromolecules were responsible for alterations in BCR trafficking, the effects of protein and carbohydrate supplementation on serum deprived B cells was assessed. Supplementation of B cell culture media with the protein BSA (68 kDa) or with the complex carbohydrate dextrans (70 kDa) was sufficient to normalize BCR surface levels (Fig. 4B). The osmolarity and viscosity of the media alone or supplemented with the various macromolecules was monitored and shown to not change (Supplemental Fig. 1H and I). Consistent with the serum fractionation, larger macronutrients or macromolecules were more effective at restoring surface BCR levels as shown by supplementing media with different sized proteins: BSA, OVA (45 kDa), or HEL (14 kDa) (Fig. 4C) or dextrans (Supplemental Fig. 1J). Denatured or fatty acid-free BSA also restored surface BCR levels, suggesting protein conformation or bound fatty acids were not critical (Supplemental Fig. 1K). While BSA supplementation did not alter MHCII surface expression, this macronutrient restored BCR internalization and MHCII presentation of BCR epitopes (Fig. 4D-F). Furthermore, MHCII presentation of an epitope derived from MHC class I heavy chain, another membrane Ag that utilizes clathrin-mediated endocytosis, was significantly impaired during serum deprivation and could be rescued by macronutrient supplementation (Fig. 4G). BSA supplementation only marginally reduced MHCII presentation of MP1 epitopes (Fig. 4H). Thus, B cell sensing of exogenous macronutrients effectively altered membrane internalization impacting BCR trafficking and MHCII peptide selection.

## Macronutrient Deprivation Impairs Trafficking and MHCII Presentation of a Cytoplasmic Antigens Which are Targets of CMA

To further elucidate the mechanism by which macronutrient deprivation altered MHCII peptide presentation, the effect of serum deprivation on MHCII presentation of glutamic acid decarboxylase (GAD), a cytosolic Ag that utilizes CMA to encounter MHCII, was monitored in B cells. Interestingly, GAD Ag mirrored the results obtained with the BCR epitope. While MHCII presentation of GAD peptide was unaltered by serum deprivation, MHCII presentation of an epitope derived from the endogenous GAD Ag was significantly impaired during macronutrient deprivation (Fig. 5A and B). Similar results were observed for MHCII presentation of a second CMA substrate, SMA (Supplemental Fig. 1L). Exogenous BSA supplementation could prevent these changes in GAD presentation (Fig. 5A). Similar to the BCR, GAD protein levels rose during serum deprivation irrespective of cycloheximide, consistent with impaired GAD degradation (Fig. 5C). GAD is degraded by the proteasome as well as lysosomal cathepsins (*27*). Serum deprivation induced proteasome activity (Fig. 5D). GAD, while a cytosolic protein, can be palmitoylated and associate with membranes (*28*). Serum deprivation altered the subcellular distribution of GAD, reducing the relative levels of free cytosolic GAD and increasing the levels of membrane-associated GAD (Fig. 5E, F). During CMA, select proteins are translocated from the cytosol to the lumen of the lysosome by the transmembrane protein LAMP2A. LAMP2 protein and mRNA levels were assessed revealing LAMP2 levels were increased by cellular serum deprivation, suggesting the expression of this transporter was not limiting (Figure 5G and H) (*29*). While epitopes from GAD and the BCR utilize seemingly disparate pathways to encounter MHCII presentation, serum deprivation altered their trafficking, degradation, and MHCII presentation in similar ways.

### Macronutrient Deprivation Impairs HSC70 Association with Cytoplasmic GAD Antigen

HSC70 is required for both clatherin-mediated endocytosis and CMA, the pathways required for BCR and GAD presentation by MHCII, respectively. To assess whether serum deprivation altered HSC70 activity, GAD association with HSC70 was monitored in B cells. GAD association with HSC70 in control serum-feed cells or BSA supplemented cells was similar, yet macronutrient deprivation significantly reduced the association of GAD and HSC70 (Fig. 6A and B). Total cellular HSC70 protein expression remained unaltered by macronutrient deprivation (Fig. 6C and D). These data suggest macronutrient deprivation may alter the subcellular distribution and function of HSC70.

### Ectopic Expression of HSC70 Overcomes the Effects of Macronutrient Deprivation in B Cells

To assess the role of HSC70 during serum deprivation, B cells were transduced to ectopically express cytoplasmic HSC70 (Fig. 7A). Ectopic expression of HSC70 restored GAD association with HSC70 during macronutrient deprivation (Fig. 7B). Though this approximately 2-fold overexpression of HSC70 did not alter expression of MHCII components, it restored MHCII presentation of epitopes derived from GAD, BCR, and MHCI during nutrient deprivation (Fig. 7C and D and Supplemental Fig. 2A-D). Furthermore, ectopic expression of HSC70 reduced MHCII presentation of the MP1 epitope in serum deprived cells (Fig. 7E). Consistent with this, ectopic expression of HSC70 prevented MA induction during serum deprivation, though surprisingly media supplementation with BSA failed to alter cellular MA flux (Supplemental Fig. 2E). Finally, ectopic HSC70 expression restored the trafficking of the BCR, TfR and GAD in B cells as seen by the normalization of surface BCR and TfR expression and restored the distribution of cytosolic and membrane GAD during macronutrient deprivation (Fig. 7F-H and Supplemental Fig. 2F). Restoration of Ag trafficking with increased HSC70 expression in B cells, demonstrates a key role for this chaperone in modulating cellular stress and intracellular protein transport.

## Discussion

In the current study, macronutrient deprivation was shown to directly impact epitope selection and MHC II Ag presentation by disrupting clathrin-mediated endocytosis and perturbing multiple routes of autophagy. Alterations in intracellular Ag trafficking compromised MHCII presentation of epitopes derived from the BCR, MHCI and the cytoplasmic Ags GAD and SMA. By contrast, MHCII display of epitopes derived from Ags targeted to autophagosomes increased with enhanced cellular MA during macronutrient deprivation. These data suggest coordinate regulation during cell stress of the pathways that deliver membrane and cytoplasmic Ags to the endosomal network for processing and MHCII presentation.

MA and clathrin-mediated endocytosis share several key chaperones including mAtg9, TBC1D5, and AP2, suggesting potential coordinate regulation of these intracellular transport pathways in different cells although this has not been examined in the context of immune cells (*30*-*32*). The current study reveals a novel role for HSC70 in controlling these

pathways during macronutrient stress of B lymphocytes. HSC70 is a constitutively expressed chaperone critical for clathrin-mediated endocytosis, proteasome degradation as well as the selective capture of proteins for translocation into lysosomes via CMA (*33*, *34*). HSC70 guided transit of proteins to the proteasome and CMA appears widely conserved in neural cells as well as immune cells (*19*, *27*, *35*, *36*). Studies from our laboratory demonstrated HSC70 binds to select proteins such as GAD Ag, delivering them for proteolytic processing and MHCII presentation (*19*, *27*). Furthermore, in fibroblasts HSC70 association with autophagosomes may play a role in the clearance of cytoplasmic protein aggregate complexes by MA (*37*, *38*). Intracellular competition for HSC70 was recently observed, as induction of MA by protein aggregates depleted chaperone reserves impairing clathrin-mediated endocytosis (*38*). Our data indicate during macronutrient stress in B lymphocytes, these trafficking pathways compete for conserved chaperones such as HSC70; with upregulation of MA and proteasome activity at the expensive of endocytosis and CMA. This may favor the presentation of intracellular pathogens or self Ags sequestered in autophagosomes, while limiting immune recognition of endocytosed Ags. Furthermore, studies here are the first to suggest a role for HSC70 in regulating stress induced MA. Thus, HSC70 plays a key role selectively regulating endocytosis and autophagy during nutritional stress, impacting Ag presentation and B-T cell interactions.

Multiple mechanisms likely contribute to compromised adaptive immune responses in malnourished individuals (*5*, *39*). For example, zinc deficiency is linked to protein malnourishment and can impair T cell responses, suggesting a role for this micronutrient in regulating host immunity (*1*, *40*). Furthermore, studies have revealed protein deprivation reduces the capacity of macrophages to phagocytosis bacteria in vivo and in non-immune cells disrupts transferrin receptor endocytosis, indicative of more global effects of nutritional stress on vesicular sorting (*32*, *41*). Whether protein malnourishment impacts Ag presenting cells such as B cells had not been tested. The current study now reveals a novel link between exogenous macronutrient levels and Ag trafficking in B cells, offering further explanation for reduced antibody responses and impaired T-cell mediated immunity observed in protein malnourished individuals (*4*). These observations also point to the exquisite sensitivity of lymphocytes to nutrient stress.

In summary, connections between cellular endocytic and autophagic pathways shape MHCII Ag presentation to influence adaptive immunity. Here, macronutrient availability was shown to upregulate MA flux and impair the ability of B cells to internalize cell surface Ag receptors, impacting MHCII Ag presentation. These data revealed HSC70 as a connection between routes of Ag delivery, suggesting coordinated regulation of these pathways may influence MHCII epitope selection. Together these findings reveal a previously unknown connection between macronutrient sensing and Ag trafficking within lymphocytes, which may contribute to impaired adaptive immune response in protein malnourished individuals.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **CMA** | Chaperone-mediated autophagy |
| **CQ** | Chloroquine |
| **CHX** | Cycloheximide |
| **GAD** | Glutamic acid decarboxylase |
| **HEL** | Hen egg lysozyme |
| **MA** | Macroautophagy |
| **MHCI** | MHC class I molecules |
| **MHCII** | MHC class II molecules |
| **MP1** | Influenza matrix protein 1 |
| **PG** | PriessGAD |
| **TfR** | transferrin receptor |

## References

1. Hansen MA, Fernandes G, Good RA. Nutrition and immunity: the influence of diet on autoimmunity and the role of zinc in the immune response. Annual review of nutrition. 1982; 2:151–177.
2. Scrimshaw NS, Taylor CE, Gordon JE. Interactions of nutrition and infection. Monograph series. World Health Organization. 1968; 57:3–329. [PubMed: 4976616]
3. Taiwo OO, Thomas KD. Plasma biochemical parameters in Nigerian children with protein energy malnutrition. East African medical journal. Aug.1992 69:428–432. [PubMed: 1396208]
4. Cripps AW, Otczyk DC, Barker J, Lehmann D, Alpers MP. The relationship between undernutrition and humoral immune status in children with pneumonia in Papua New Guinea. Papua and New Guinea medical journal. Sep-Dec;2008 51:120–130. [PubMed: 21061943]
5. Najera O, Gonzalez C, Toledo G, Lopez L, Ortiz R. Flow cytometry study of lymphocyte subsets in malnourished and well-nourished children with bacterial infections. Clinical and diagnostic laboratory immunology. May.2004 11:577–580. [PubMed: 15138185]
6. Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Muller M, Kreymborg K, Altenberend F, Brandenburg J, Kalbacher H, Brock R, Driessen C, Rammensee HG, Stevanovic S. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proceedings of the National Academy of Sciences of the United States of America. May 31.2005 102:7922–7927. [PubMed: 15894616]
7. Crotzer VL, Blum JS. Cytosol to lysosome transport of intracellular antigens during immune surveillance. Traffic. Jan.2008 9:10–16. [PubMed: 17916226]
8. DeFranco AL, Richards JD, Blum JH, Stevens TL, Law DA, Chan VW, Datta SK, Foy SP, Hourihane SL, Gold MR, et al. Signal transduction by the B-cell antigen receptor. Annals of the New York Academy of Sciences. Sep 7.1995 766:195–201. [PubMed: 7486656]

9. McGovern EM, Moquin AE, Caballero A, Drake JR. The effect of B cell receptor signaling on antigen endocytosis and processing. Immunological investigations. May.2004 33:143–156. [PubMed: 15195694]
10. Davidson HW, West MA, Watts C. Endocytosis, intracellular trafficking, and processing of membrane IgG and monovalent antigen/membrane IgG complexes in B lymphocytes. J Immunol. Jun 1.1990 144:4101–4109. [PubMed: 2187925]
11. Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. Nature. Dec 5.1991 354:392–394. [PubMed: 1956401]
12. Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. Cell. Jul 14.1995 82:155–165. [PubMed: 7606781]
13. Drake L, McGovern-Brindisi EM, Drake JR. BCR ubiquitination controls BCR-mediated antigen processing and presentation. Blood. Dec 15.2006 108:4086–4093. [PubMed: 16931624]
14. Watts C, Davidson HW. Endocytosis and recycling of specific antigen by human B cell lines. The EMBO journal. Jul.1988 7:1937–1945. [PubMed: 3262056]
15. Stipanuk MH. Macroautophagy and its role in nutrient homeostasis. Nutrition reviews. Dec.2009 67:677–689. [PubMed: 19941614]
16. Cuervo AM, Knecht E, Terlecky SR, Dice JF. Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation. The American journal of physiology. Nov.1995 269:C1200–1208. [PubMed: 7491910]
17. Wicker LS, Chen SL, Nepom GT, Elliott JF, Freed DC, Bansal A, Zheng S, Herman A, Lernmark A, Zaller DM, Peterson LB, Rothbard JB, Cummings R, Whiteley PJ. Naturally processed T cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes-associated human MHC class II allele, DRB1*0401. The Journal of clinical investigation. Dec 1.1996 98:2597–2603. [PubMed: 8958223]
18. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity. Jan.2007 26:79–92. [PubMed: 17182262]
19. Zhou D, Li P, Lin Y, Lott JM, Hislop AD, Canaday DH, Brutkiewicz RR, Blum JS. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity. May.2005 22:571–581. [PubMed: 15894275]
20. Haque MA, Li P, Jackson SK, Zarour HM, Hawes JW, Phan UT, Maric M, Cresswell P, Blum JS. Absence of gamma-interferon-inducible lysosomal thiol reductase in melanomas disrupts T cell recognition of select immunodominant epitopes. The Journal of experimental medicine. May 20.2002 195:1267–1277. [PubMed: 12021307]
21. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy. Nov-Dec;2007 3:542–545. [PubMed: 17611390]
22. Kirschke H, Wiederanders B. Cathepsin S and related lysosomal endopeptidases. Methods in enzymology. 1994; 244:500–511. [PubMed: 7845228]
23. Creasy BM, Hartmann CB, White FK, McCoy KL. New assay using fluorogenic substrates and immunofluorescence staining to measure cysteine cathepsin activity in live cell subpopulations. Cytometry. Part A : the journal of the International Society for Analytical Cytology. Feb.2007 71:114–123. [PubMed: 17200959]
24. Moravec R, O'Brien M, Daily B, Scurria M, Bernad L, Larson B, Worzella T, Rashika K, Culp J, McNamara B, Riss T. Monitoring Proteasome Activity with a Cell-Based Assay Using a Single-Addition Luminescent Method. Cell Notes. 2006; 1
25. Turvy DN, Blum JS. Detection of biotinylated cell surface receptors and MHC molecules in a capture ELISA: a rapid assay to measure endocytosis. Journal of immunological methods. Mar 1.1998 212:9–18. [PubMed: 9671148]
26. Christgau S, Schierbeck H, Aanstoot HJ, Aagaard L, Begley K, Kofod H, Hejnaes K, Baekkeskov S. Pancreatic beta cells express two autoantigenic forms of glutamic acid decarboxylase, a 65-kDa hydrophilic form and a 64-kDa amphiphilic form which can be both membrane-bound and soluble. The Journal of biological chemistry. Nov 5.1991 266:21257–21264. [PubMed: 1939164]

27. Lich JD, Elliott JF, Blum JS. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. The Journal of experimental medicine. May 1.2000 191:1513–1524. [PubMed: 10790426]
28. Kanaani J, Patterson G, Schaufele F, Lippincott-Schwartz J, Baekkeskov S. A palmitoylation cycle dynamically regulates partitioning of the GABA-synthesizing enzyme GAD65 between ER-Golgi and post-Golgi membranes. Journal of cell science. Feb 15.2008 121:437–449. [PubMed: 18230651]
29. Cuervo AM, Dice JF. Regulation of lamp2a levels in the lysosomal membrane. Traffic. Jul.2000 1:570–583. [PubMed: 11208145]
30. Longatti A, Tooze SA. Recycling endosomes contribute to autophagosome formation. Autophagy. Nov.2012 8:1682–1683. [PubMed: 22874560]
31. Popovic D, Dikic I. TBC1D5 and the AP2 complex regulate ATG9 trafficking and initiation of autophagy. EMBO reports. Apr 1.2014 15:392–401. [PubMed: 24603492]
32. Puri C, Renna M, Bento CF, Moreau K, Rubinsztein DC. Diverse autophagosome membrane sources coalesce in recycling endosomes. Cell. Sep 12.2013 154:1285–1299. [PubMed: 24034251]
33. McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nature reviews. Molecular cell biology. Aug.2011 12:517–533.
34. Agarraberes FA, Dice JF. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. Journal of cell science. Jul.2001 114:2491–2499. [PubMed: 11559757]
35. Elliott E, Tsvetkov P, Ginzburg I. BAG-1 associates with Hsc70.Tau complex and regulates the proteasomal degradation of Tau protein. The Journal of biological chemistry. Dec 21.2007 282:37276–37284. [PubMed: 17954934]
36. Zhang C, Cuervo AM. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. Nature medicine. Sep.2008 14:959–965.
37. Olzscha H, Schermann SM, Woerner AC, Pinkert S, Hecht MH, Tartaglia GG, Vendruscolo M, Hayer-Hartl M, Hartl FU, Vabulas RM. Amyloid-like aggregates sequester numerous metastable proteins with essential cellular functions. Cell. Jan 7.2011 144:67–78. [PubMed: 21215370]
38. Yu A, Shibata Y, Shah B, Calamini B, Lo DC, Morimoto RI. Protein aggregation can inhibit clathrin-mediated endocytosis by chaperone competition. Proceedings of the National Academy of Sciences of the United States of America. Apr 15.2014 111:E1481–1490. [PubMed: 24706768]
39. Forte WC, Santos de Menezes MC, Horta C, Carneiro Leao Bach R. Serum IgE level in malnutrition. Allergologia et immunopathologia. Mar-Apr;2003 31:83–86. [PubMed: 12646123]
40. Fraker PJ, King LE. Reprogramming of the immune system during zinc deficiency. Annual review of nutrition. 2004; 24:277–298.
41. Redmond HP, Gallagher HJ, Shou J, Daly JM. Antigen presentation in protein-energy malnutrition. Cellular immunology. Jun.1995 163:80–87. [PubMed: 7758132]

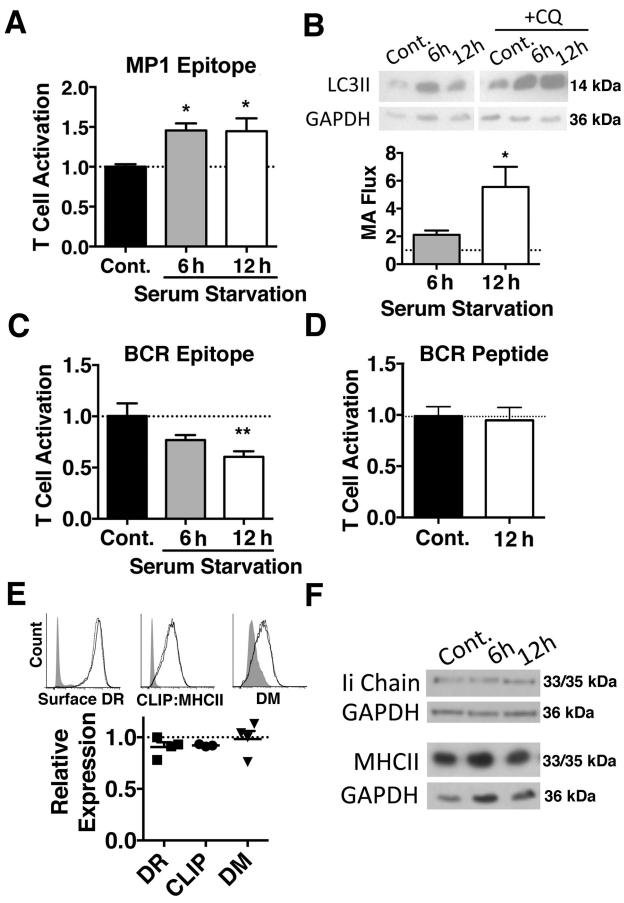


**Figure 1. Macronutrient deprivation alters endogenous Ag presentation and degradation**

(**A**) B cells were grown + serum (Cont.) or − serum and MHCII presentation of MP1 epitopes was monitored. (**B**) To measure MA flux, B cells were grown +/− serum and treated with CQ to stabilize and detect endogenous LC3II accumulation in autophagosomes. MA flux in Cont. cells was normalized and set to 1 (dotted line). (**C**) B cells were grown +/− serum and MHCII presentation of endogenous BCR epitopes detected using epitope-specific T cells. (**D**) B cells were cultured +/− serum for 12 h, fixed and incubated for 6 h with 10 uM BCR peptide prior to incubation with T cells and detection of Il-2. (**E**) B cells were grown for 12 h +/− serum and changes in the expression of surface HLA-DR (MHCII) and intracellular HLA-DM detected by flow cytometry. HLA-DM function was assessed by monitoring MHCII:CLIP surface levels. Protein levels in Cont. cells were set to 1 (dotted line) and the relative expression of DR, CLIP or DM during serum deprivation indicated. (**F**) B cells were grown +/− serum for 12 h and lysed for immunoblot analysis of MHCII, invariant chain, and GAPDH. (**A and C**) One-way ANOVA, multiple comparison. (**B**) Student's T-Test, multiple comparison to Cont. cells.

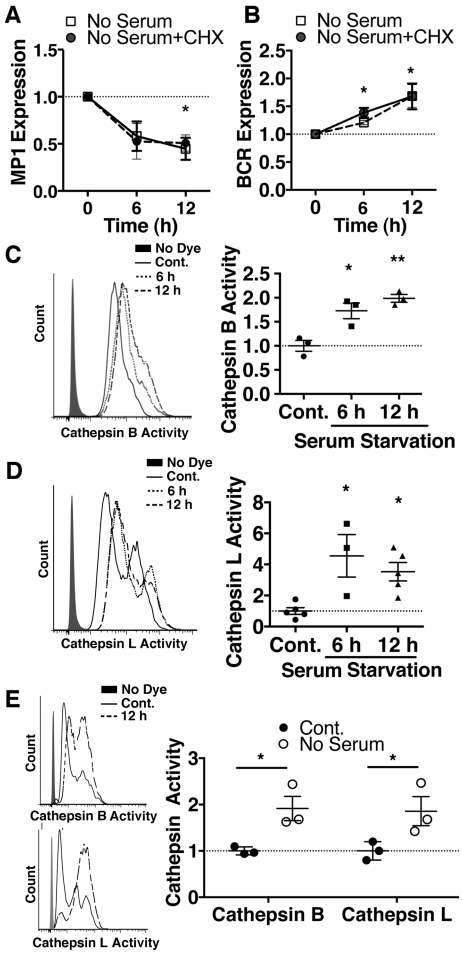


**Figure 2. Macronutrient deprivation inhibits BCR turnover while activating endosomal/lysosomal proteases**

(**A and B**) B cells were grown + serum (Cont.) or − serum and +/− cycloheximide (CHX). MP1 and BCR protein expression were determined relative to cellular GAPDH. Protein expression in Cont. cells was normalized and set to 1 (dotted line). (**C and D**) Real-time analysis of cathepsin B and L activity in B cells cultured as Cont. or without serum for 6 or 12 h. Fluorescence profile for no substrate treated cells is shown (gray shaded). (**E**) Real-time assessment of cathepsin B and L activity in human peripheral blood B cells treated as Cont. or without serum for 12 h. (**A and B**) Student's T-Test, multiple comparison to Cont. cells. (**C and D**) One-way ANOVA, multiple comparison. (**E**) Two-way ANOVA, multiple comparison.

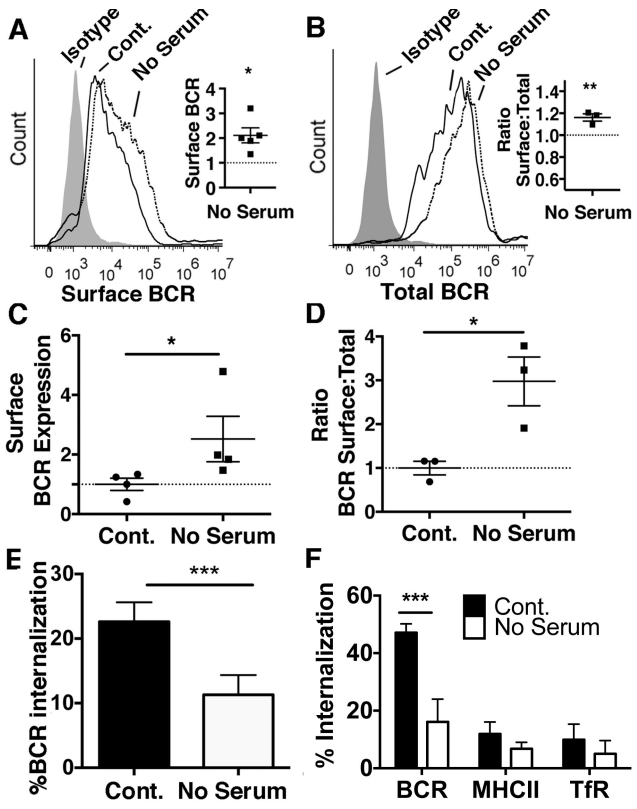


**Figure 3. Macronutrient deprivation alters BCR trafficking**

Flow cytometric analysis of B cells cultured + serum (Cont.) or − serum for 12 h prior to detection of surface or total BCR levels. (**A)** Surface BCR levels (insert: relative surface levels of BCR for B cells cultured without serum vs. Cont. cells indicated by the dotted line). (**B**) Total BCR levels (insert: relative ratio of surface:total BCR for cells treated with no serum vs. Cont. cells indicated by the dotted line). Fluorescence profile for cells stained with an isotype matched control Ab is shown as gray shaded area. (**C and D**) Peripheral blood B cells were treated +/− serum for 12 h and stained for BCR surface levels (**C**) and the relative ratio of surface:total BCR **(D)**. For panels A-D flow cytometric data for Cont. cells was set equal to 1. (**E)** BCR endocytosis was monitored in B cells cultured +/− serum. (**F**) Specific endocytosis of the BCR, MHCII or transferrin receptor (TfR) was monitored in peripheral blood B cells incubated +/− serum. (**A-F**) Student's T-Test.

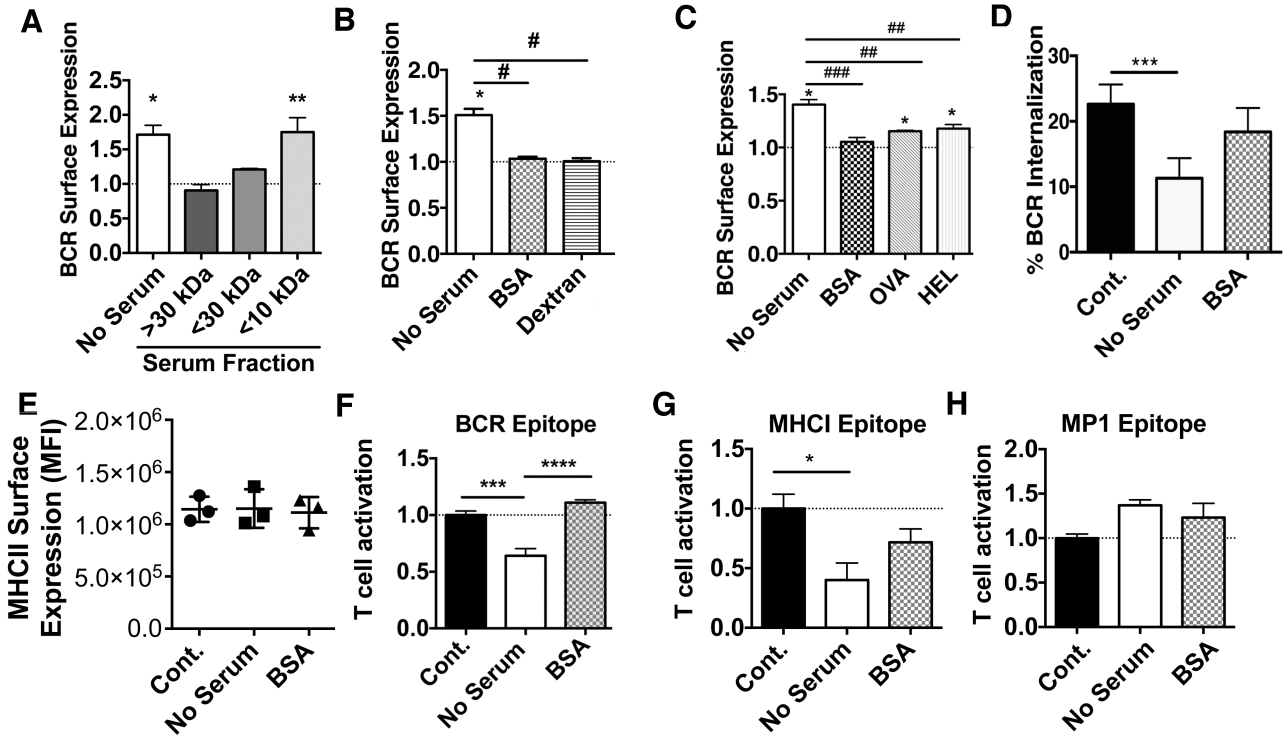


**Figure 4. Exogenous macromolecules restore BCR trafficking and MHCII Ag presentation**

(**A**) BCR surface expression was determined in B cells treated for 12 h + serum (Cont.), − serum, or in serum-free media supplemented with different fractions of serum (>30kDa, <30kDa, or <10kDa). Cont. cell BCR surface expression was normalized and set to 1 (dotted line). (**B**) BCR surface expression was determined in B cells treated for 12 h +/− serum, or in serum-free media supplemented with BSA or 70 kDa dextran. Cont. cell BCR surface expression was normalized and set to 1 (dotted line). (**C**) BCR surface levels were determined in B cells cultured +/− serum or in serum-free media supplemented with various sized proteins: 14 kDa HEL, 45 kDa OVA, or 68 kDa BSA for 12 h. (**D**) BCR endocytosis was monitored in B cells cultured +/− serum or in serum-free media supplemented with BSA. (**E**) B cells were cultured +/− serum, or in serum-free media supplemented with BSA for 12 h, harvested and fixed for flow cytometric analysis of MHCII surface expression. MHCII presentation of (**F**) BCR epitopes, (**G**) MHC class I (MHCI) epitopes, and (**H**) MP1 epitopes was monitored in B cells treated +/− serum or in serum-free media supplemented with BSA for 12 h. (**A**-**C**) Student's T-Test, multiple comparison * Denotes p value as compared to Cont. cells (dotted line). # denotes a One-way ANOVA, multiple comparison. (**D**-**H**) One-way ANOVA, multiple comparison.

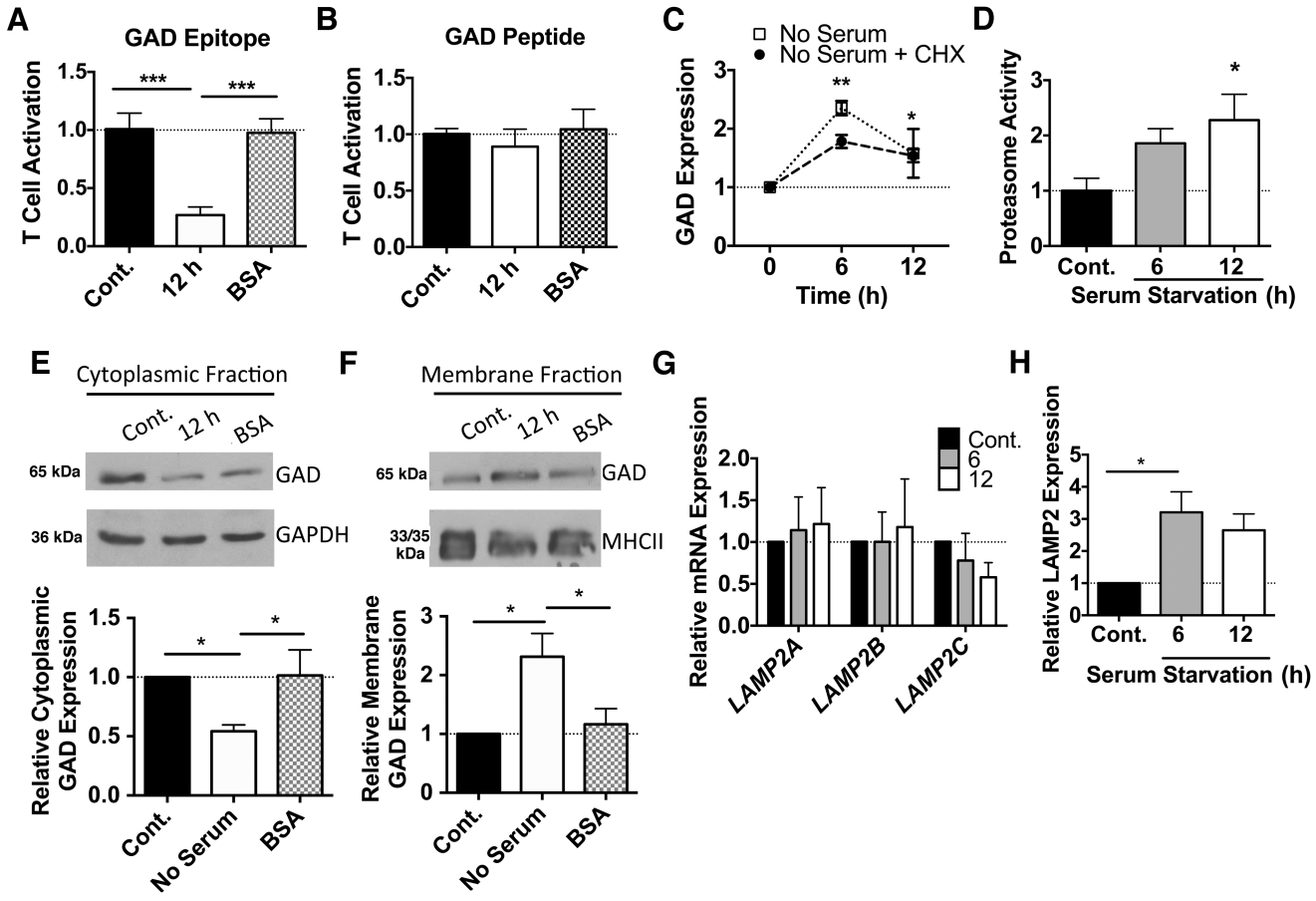


**Figure 5. Macronutrient deprivation alters trafficking and MHCII presentation of the endogenous GAD Ag**

(**A and B**) B cells were grown + serum (Cont.) or − serum and MHCII presentation of endogenous GAD epitopes (**A**) or exogenous GAD peptide (**B**) was monitored. (**C**) B cells were grown +/− serum and +/− cycloheximide (CHX). Cellular GAD protein expression was determined relative to GAPDH. Protein expression in Cont. cells was normalized and set to 1 (dotted line). (**D**) Proteasome activity was monitored in B lymphoblasts grown +/− serum for 6 or 12 h using a luminescent substrate. (**E and F**) B cells were grown +/− serum or in media supplemented with BSA for 12 h, harvested, and lysed into cytoplasmic and membrane fractions. Lysates were resolved by SDS-PAGE and immunobloted for GAD and as a loading control GAPDH (cytoplasmic) or MHCII (membrane). (**G**) B cells were cultured +/− serum for up to 12 h and the mRNA levels of the LAMP2 isoforms or (**H**) cellular LAMP2 protein expression was assessed. **(A, D-F and H**) One-way ANOVA, multiple comparisons. (**C**) Student's T-Test, multiple comparison to Cont. cells.

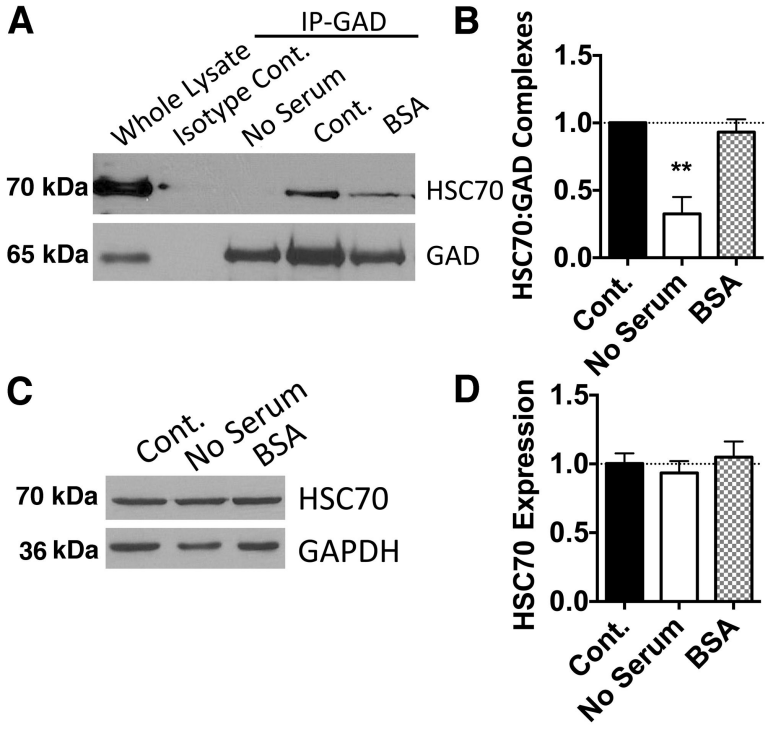


**Figure 6. Macronutrient deprivation alters HSC70 association with endogenous Ags**

(**A**) B cells were cultured + serum (Cont.), − serum, or in serum-free media supplemented with BSA, harvested and lysed. Lysates were incubated with GAD Ab or an isotyped-matched Ab overnight and protein G-Sephorose beads the next day. Immunoprecipitated proteins were resolved using SDS-PAGE, immunoblotted for HSC70 and GAD, and (**B**) densitometry was used to quantitate relative amounts of HSC70 bound to GAD. (**C**) B cells were grown +/− serum or in serum-free media supplemented with BSA for 12 h and lysed for immunoblot analysis of HSC70 and GAPDH. (**D**) Densitometry was used to quantitate relative ratios of HSC70 to GAPDH. Student's T-Test, multiple comparisons.

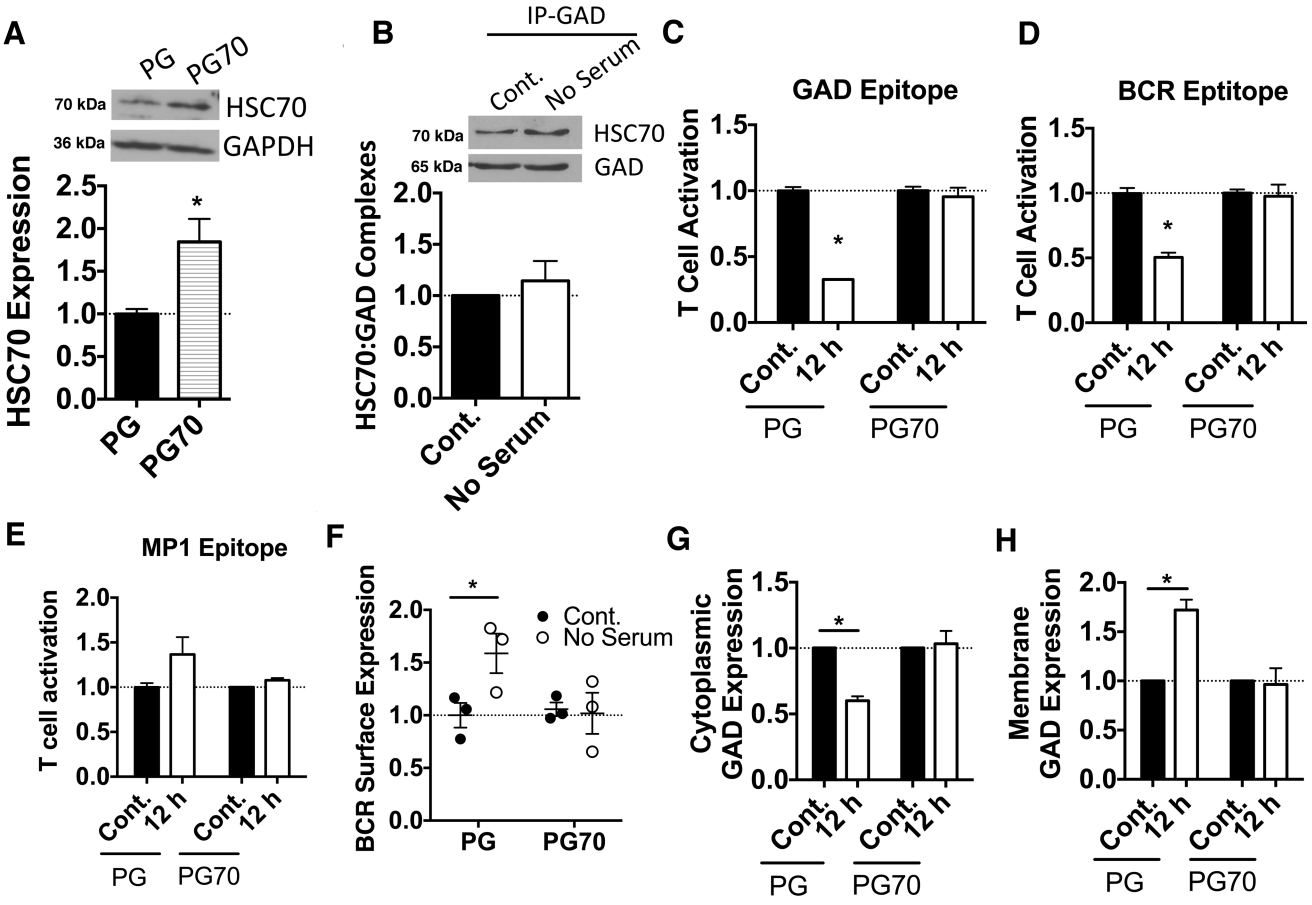


**Figure 7. Ectopic expression of HSC70 averts disruptions in Ag trafficking during macronutrient deprivation**

(**A**) The PriessGAD (PG) B lymphobasts were transfected to ectopically express HSC70 (PG70). PG and PG70 cells were lysed for immunoblot analysis of HSC70 and GAPDH expression. Densitometry was used to quantitate relative ratio of HSC70 to GAPDH expression in these cells. (**B**) PG70 cells were cultured + serum (Cont.) or − serum for 12 h. Lysates from these cells were incubated with a GAD-specific Ab or an isotyped-matched Ab overnight to immunoprecipitate GAD. Immunoprecipitated proteins were resolved using SDS-PAGE, immunoblotted for HSC70 and GAD, and densitometry was used to quantitate relative amounts of HSC70 bound to GAD. (**C**) PG and PG70 cells were grown +/− serum and tested for MHCII presentation of endogenous GAD epitopes, (**D**) endogenous BCR epitopes, or (**E**) MP1 epitopes. (**F**) Flow cytometry of PG and PG70 cells cultured +/− serum for 12 h prior to detection of BCR surface levels. (**G and H**) PG70 cells were grown +/− serum or in media supplemented with BSA for 12 h. These cells were lysed and cytoplasmic and membrane fractions isolated. Fractions were resolved by SDS-PAGE and immunobloted for GAD and as a loading control GAPDH (cytoplasmic) or MHCII (membrane). Densitometry was used to quantitate GAD levels relative to the loading control protein. (**A**) Student's T-test. (**C-H**) Student's T-Test, multiple comparisons correction.